ORGAN CULTURE OF HUMAN SOMATOTROPHIC PITUITARY ADENOMAS: ULTRASTRUCTURE AND GROWTH HORMONE PRODUCTION

By

F. Peillon, M. Gourmelen, M. Donnadieu, A. Brandi, D. Sevaux and M. T. Pham Huu Trung

ABSTRACT

Ten somatotrophic adenomas removed from acromegalic patients and fragments of the non-tumoural surrounding pituitary were submitted to organ culture for periods of up to one month. Electron microscopic observation shows that these tumours retain their histological differentiation throughout the culture period. The cell morphology of the cultured tumours remains essentially unchanged and in particular the secretory granules keep their initial size (150 and 130 nm). However the granules disappear gradually so that most of the cells look chromophobic by the 4th week of culture, and numerous lysosomes as well as autophagic figures appear at the same time. The hGH concentration in the culture medium has been measured of 4 adenomas. It is very high (10-fold greater than that from non-tumoural pituitary medium) during the first week (range 200–300 μg/ml). It still remains very high in the same experiments until the second week of culture in one experiment (200 μg/ml). After incubation of cultures with 3H-leucine, 3H-hGH is obtained in the medium giving evidence of hormone synthesis by adenoma cells in culture. 3H-hGH represents 40% of 3H-proteins in the culture medium and gives the same elution pattern as standard hGH on Sephadex G 100 chromatography. A certain degree of correlation is observed between morphological and biological results: the greatest hGH production is obtained from explants which maintain the best histological appearance.
Organ culture represents a relatively simple system for the study of normal or abnormal pituitaries in the absence of hormonal or neurogenic control factors. Many experiments have been performed on anterior pituitary function *in vitro* especially in animals, but morphological studies are scarce, particularly those of normal or abnormal human pituitary in organ culture.

Human somatotrophic adenomas and some other types of pituitary tumours (chromophobic or prolactin secreting) are suitable for organ culture because they can be obtained easily during surgical removal and their structure and hormonal secretion are well known.

It has already been demonstrated that explant and monolayer cultures of pituitary adenomas from acromegalic patients continue to produce measurable levels of immuno-reactive growth hormone for periods as long as one year (Kohler et al. 1969). In these *in vitro* systems labelled hGH was obtained after incorporation of radioactive amino acids giving evidence for *de novo* synthesis of hormone (Kohler et al. 1971).

The current study was undertaken in order to determine whether

- adenomas in organ culture maintain the cytological differentiation of adenomas *in vivo*.
- somatotrophic cells in organ culture are similar to those seen in intact adenomas.
- correlation may be established between morphological and biological results.

**MATERIAL AND METHODS**

Pituitary adenomas were removed surgically from 10 acromegalic patients. The diagnosis was established on clinical, radiological and biological criteria. Tumour tissue was immediately placed into culture medium (M 199 supplemented with 20% foetal calf serum and 2000 U/ml penicillin) and was subsequently divided into fragments, approximately 0.5 mm in size. The organ culture was then performed according to a method described previously (Lasnitzki 1964). Five explants were mounted with cataract knives onto a strip of moistened lens paper and two such strips arranged on a grid of stainless steel. The grid supporting 10 explants was placed in a culture chamber of borosilicate glass of 30 mm in diameter which was filled with culture medium up to level of the grid, usually 1.5 ml. Two culture chambers rested in a Petri dish of 90 mm in diameter, carpeted with several layers of moistened filter paper to avoid evaporation. All the explants were incubated at 37°C in air and the culture medium was changed every 3 days for a period of 14 or 28 days. The number of culture chambers used for one single adenoma varied according to the weight of the tumour. But in all cases the number and the size of the explants were identical for one culture chamber.

Fragments of non-tumoural pituitary tissue surrounding adenoma were also obtained from 2 patients and placed in culture in a similar manner.
A – Morphological studies

Explants were studied by light and electron microscopy at the 7th, 14th, 21st and 28th day of culture. For the light microscopic studies, explants were fixed with Gerard's fluid imbedded in wax and stained with Herlant's tetrachrome. For the electron microscopic studies, the explants were fixed in glutaraldehyde (2.5% glutaraldehyde) in a buffered solution of NaCl and phosphate at pH 7.2 with an osmolarity of 300 milliosmoses; post-fixed with osmium tetroxide and imbedded in Epon (Laft 1961). Selections were cut with L.K. ultra-microtome and stained with uranyl acetate followed by lead citrate (Reynolds 1963).

B – Hormonal studies

Media removed from cultures of 4 adenomas (with different series for each of them) were frozen and later assayed for hGH. The hGH determination was performed on the media corresponding to the explants studied by light and electron microscopy. Radioimmunoassay of hGH was described previously (Donnadieu et al. 1968): antibodies were obtained by immunizing guinea pigs with hGH extracted by Raben's procedure; the hGH used at a tracer was extracted by Ross’ procedure and was purified by chromatography on Sephadex G 100 after iodination; the standard was hGH standard from “London Institute for Medical Research” (1st IRP Growth Hormone hu/h immun 66/217), diluted in barbitone-acetate buffer containing bovine serum albumin (0.1%). The culture medium was diluted (1:102 to 1:105) in the same buffer until the measured quantities of hGH in the tube were in the range 0.05–1.6 ng; a second assay with two dilutions was then performed to check the parallelism of curves and to increase the precision.

3H-leucine incorporation. – In two experiments 23 μCi of 3H-leucine ([3H-4,5] L-leucine, 15 Ci/μmol from CEA France) was added to 1.5 ml of modified medium 199 containing neither foetal calf serum nor leucine. After complete removal of the culture medium, the 3H-leucine solution was added to the cultures at the 7th and 14th day of culture respectively; 24 h later 0.5 ml of complete medium was added. After an additional 24 h incubation, the medium was removed and frozen for hGH radioimmunooassay and 3H-leucine incorporation study.

3H-proteins determination. – Proteins were precipitated by trichloroacetic acid (TCA) (4 ml of a 15% solution plus 1 ml of medium and 0.2 ml of a 20 mg/ml calf serum albumin solution). After washing twice with TCA, the precipitate was dissolved in 1 ml of Tris-HCl buffer (0.01 𝜇mol pH 8.2) added to 20 ml of NaOH-9 n, precipitated again by TCA and then washed in ether, and finally dissolved in 1 ml of soluene and counted in a liquid scintillation counter. Radioactive measurements were corrected to absolute radioactivity.

3H-hGH determination. – An excess of guinea pig anti-hGH serum was added to the media and the antibody-antigen complexes separated by goat anti-guinea pig serum fixed on solid phase (DASP). The immunoprecipitate was washed several times, dissociated by HC1, centrifuged, and the proteins of the supernatant precipitated with TCA and treated as above. One ng 125I-hGH was used as a tracer. The same experiment was made without anti-hGH antibody to determine non-specific adsorption of proteins on the DASP. A possible adsorption on DASP of 3H-leucine added to identical culture medium (unexposed to culture cell) was ruled out.
hGH extraction. – An extraction was performed from the explants of two series of the same adenoma at the end of the culture period, and from a fragment of one adenoma frozen immediately after surgery. The technique used was the procedure of Raben (1957) applied to plasma by Hunter & Greenwood (1964). hGH was measured on these extracts by radioimmunoassay.

RESULTS

A – Morphology

a) Light microscopy. – After 2 or 3 days the explants become round or oval and are surrounded by a border of 2 or 3 layers of epithelial-like cells. The pituitary fragments show no tendency to spread, neither glandular cells nor fibroblasts being observed. On the contrary explants become smaller after 7 days of culture, then keep the same diameter during the following 3 weeks of culture. In the centre of the explants, there are clusters of necrotic cells with dark condensed nuclei. These cells no longer have plasma membranes but the cytoplasm remains around the pyknotic nucleus. Characteristic secretory granules are quite numerous in their cytoplasm and are eosinophilic like the granules of somatotrophic cells. The necrotic cells are surrounded by many normal somatotrophic cells which contain a large number of granules during the first 7 days of culture. Then they decrease so that the cells appear chromophobic during the ensuing 3 weeks of culture. At the same time lysosomes are increased mainly in the neighbourhood of the necrotic cells.

Fig. 1.
Portion of an intact human somatotrophic adenoma. The cells contain numerous secretory granules which are of two types: larger (350 nm large arrows) and smaller (150 nm small arrows).
b) *Electron microscopy* (Figs. 1–7). Most explants retain a well organized appearance during the first 7 days of culture. Thereafter a few remain well differentiated until the 28th day of culture. The cells of adenomas in culture, like those of normal pituitary and intact adenomas (Fig. 1) are not bound by junctional complexes. They form a rather compact parenchyma with relatively rare capillaries. The parenchymatous basal lamina are absent.

Three types of cells can be described based on the different types and number of granules. The first one contains granules similar to somatotrophic granules (300–350 nm, Figs. 2–5). The second one contains smaller granules (150 nm, Figs. 2–6) which are separated from their outer membrane by a clear space. The two types of granules can be seen in the same cell. The third type of cell contains no or very few granules (Fig. 3), and these cells are quite numerous on the 28th day of culture. When present, the granules are mainly found near the plasma membranes although an extruded granule is rarely observed. Abundant lysosomes are present, many of which display aberrant changes toward the granules (Figs. 2–5). The increasing number of lysosomes between the 7th and 28th day of culture is obvious in Figs. 2 and 3. The Golgi apparatus is prominent in all the cells and it has a great number of dictyosomes with long saccules and smooth or coated vesicles. Granules and immature lysosomes can be identified in the Golgi apparatus (Figs. 5–6). Mitochondria are numerous and their matrix is dense. In some cells there are abnormal structures composed of submicroscopic filaments, often juxta nuclear and sometimes depressing the nucleus. They are large in size (about 5 μm) and contain in their mesh some cellular organelles including secretory granules (Fig. 7).

In the necrotic cells granules are of the same size as those observed in the somatotrophic cells. They are of the two same types and are very abundant (Fig. 4).

**B – Hormonal secretion**

a) *Non-tumoural pituitary fragments* (Table 1, P<sub>B</sub>-P<sub>D</sub>). The concentration of hGH is very high in the culture medium during the first 72 h: 44 and 47 μg/ml (P<sub>B</sub>-P<sub>D</sub}); it decreases sharply in the next 4 additional days of culture. By the end of the second week, hGH levels fall to 4 and 4.05 μg/ml.

In one experiment hGH release was related to the initial weight of the pituitary fragment and the culture duration: it decreased from 16.5 ng/mg/h during the first 3 days culture period to 0.24 ng/mg/h on the 14th day.

b) *Somatotrophic adenomas* (Table 1, A-B-C-D). In the first culture medium samples, collected after 72 h, very high hGH levels are observed (Table 1, B<sub>1</sub>-B<sub>2</sub>-B<sub>3</sub>). They are 10 fold higher than that of corresponding media derived from non-tumoural pituitary fragment in the two experiments in which the
Table 1.

hGH concentration (μg/ml) in culture media from normal pituitaries and somatotrophic adenomas.

<table>
<thead>
<tr>
<th>Culture time (days)</th>
<th>Normal pituitary A</th>
<th>Somatotrophic adenomas B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_B**</td>
<td>P_D***</td>
<td>1 2</td>
<td>1 2 3</td>
</tr>
<tr>
<td>1</td>
<td>390</td>
<td>1017</td>
<td>482</td>
<td>373</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>47</td>
<td>366</td>
<td>480</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>6</td>
<td>285</td>
<td>287</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>0.5</td>
<td>173</td>
<td>99</td>
</tr>
<tr>
<td>21</td>
<td>0.2</td>
<td>1</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>28</td>
<td>0.1</td>
<td>0.2</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

* Numbers 1 to 3 represent the different organ cultures performed simultaneously from each adenoma.

** Normal pituitary tissue derived from patients B and D.

*** In one experiment, hGH was measured in the medium which the tissue was maintained between the time of surgery and the initiation of the culture (approx. 4 h).

A parallel study could be performed (Table 1, P_B-P_D). hGH concentrations remain very high exceeding 200 μg/ml in 15 of 18 samples of culture media collected on the 7th day. After this the hGH excretion decrease is very marked in some series (B_1-D) but it is slow in others (C_1-B_3). In series B_3 for example

Fig. 2.

Portion of a human somatotrophic adenoma cultivated for 7 days. The cells show a decreased number of secretory granules which have the same two sizes as in intact adenoma: 350 nm (large arrows) and 150 nm (small arrows). Lysosomes (vacuolated dense bodies) are present (L).

Fig. 3.

Portion of a human somatotrophic adenoma cultivated for 28 days. The cells contain few granules (small arrows) or no granules at all but they have an increased number of lysosomes (arrowheads).

Fig. 4.

Portion of the necrosis from human somatotrophic adenoma cultivated for 28 days. Characteristic secretory granules are observed: 350 nm (large arrows) and 150 nm (small arrows). Cells have no cytoplasmic membrane but cytoplasm remains around the nucleus (N).

223
the hGH concentration in the culture medium is the same on the 28th day and on the 7th day.

The mean production rate per mg of tumoural tissue estimated from 8 series decreases from 142 ng/mg/h (range 22–261) on the 7th day to 51 ng/mg/h (range 3.6–133) on the 14th day.

In no instance has any hGH been detected in the control medium. Addition of ovine prolactin (up to 10 µg) does not displace the hGH anti-hGH antibodies complex.

Table 2.

3H-leucine incorporation (34.10⁶ dpm/ml) into proteins of culture media from two somatotrophic adenomas.

<table>
<thead>
<tr>
<th>Culture time (days)</th>
<th>³H-proteins DPM/ml</th>
<th>³H-proteins ³H-leucine %₀</th>
<th>³H-hGH DPM/ml</th>
<th>³H-hGH ³H-proteins %₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>9°</td>
<td>4 359 500</td>
<td>12.8</td>
<td>1 759 200</td>
<td>40.0</td>
</tr>
<tr>
<td>11°</td>
<td>941 040</td>
<td>2.8</td>
<td>252 700</td>
<td>26.9</td>
</tr>
<tr>
<td>16°*</td>
<td>1 394 390</td>
<td>4.1</td>
<td>483 020</td>
<td>34.7</td>
</tr>
</tbody>
</table>

° Incorporation on the 7th day.
** Incorporation on the 15th day.

c) Pituitary extracts. – hGH contents in tissue extracts from a non-culture fragment of one adenoma was found to be 2.2 µg/mg of wet weight. After 4 weeks of culture, tissue extracts from two series of explants of the same adenoma contained 1.4 and 161 ng/mg respectively. These values were less elevated than those found in the corresponding media.

Fig. 5.
Somatotrophic adenomatous cell cultivated for 7 days. Secretory granules of 300–350 nm (large arrows) and lysosomes (vacuolated dense bodies L). A prominent Golgi apparatus (Go) is observed.

Fig. 6.
Somatotrophic adenomatous cell cultivated for 28 days. There are few secretory granules of 150 nm (small arrows) but various forms of lysosomes (arrowheads). The prominent Golgi apparatus (Go) contains granules.

Fig. 7.
Abnormal structures observed in human somatotrophic adenomas in culture, as well as in the intact one. They are composed of submicroscopic filaments containing secretory granules.
Chromatography on Sephadex G 100 (1.5 × 54 cm).
Elution with barbital buffer 0.007 M - pH 7.5. Two ml fractions were collected and analyzed for radioactivity (CPM).

**Fig. 8.**

**d) ³H-leucine incorporation.** – The total radioactivity of medium proteins is reported in Table 2. Results are expressed in DPM/ml of medium. On the 9th day of culture, 12.8% of the radioactivity added was recovered in TCA precipitate after 48 h of incubation.

The amount of radioactivity recovered by DASP was about 40% of the total radioactivity of the ³H-medium proteins.

When submitted to chromatography on Sephadex G 100, ³H-hGH obtained from culture media after addition of ³H-leucine gives the same elution pattern as standard ¹³¹I-hGH (Fig. 8).

The specific activity of labelled hormone obtained was 5.3 µCi/mg.

**DISCUSSION**

From both these morphological and biological studies, it appears that somatotrophic adenomas in organ culture are able to maintain their histological differentiation over a 4 week growth period and to synthetize and release large amounts of hGH into the medium without hypothalamic control. Somatotrophic cells studied by light and electron microscopy show the same aspects in culture as those observed in non-cultured adenomas. Their fine structure is similar and they have the same Golgi apparatus, the same mitochondria, the same submicroscopic filaments and the same two types of secretory granules. However as can be seen in Fig. 1 there are more granules in the intact adenomas than in the cultured ones. Somatotrophic adenomas have occasionally few or
even no granules and these adenomas appear to be chromophobic (Young et al. 1965; Russfield 1968; Olivier et al., in press). Most adenomas in the present study however show the following evolution in culture: a decreased number of granules seen as early as on the 7th day of culture (Fig. 2), while there is an increasing number of lysosomes and autophagic figures (Fig. 3). These two facts and the presence of necrotic cells in the middle of the explants represent the main changes observed in cultured adenomas.

The above morphological findings are highly suggestive of a continued process in the adenomas in culture. This is further substantiated by the determination of large sustained amounts of hGH in the culture media.

The following findings should be emphasized: a) The present study confirms the in vitro release and the synthesis of hGH by somatotrophic adenomas, already demonstrated by other investigators. Kohler et al. (1969) obtained hGH values between 50 and 400 ng/day culture and hGH production was sustained for up to one year. Batzdorf et al. (1971) found hGH concentration between 0.46 and 13 μg/ml in somatotrophic adenoma culture media maintained for 7 or 14 days in culture. hGH production appears important in our study and high levels are observed in some instances (range 100 to 200 μg/ml). The larger amounts of cultured tumoural tissue and the different culture methods used may explain these quantitative differences. Furthermore values of hGH in our study may be an underestimation of the potential capacity of the tissue to produce hormone. Medium was removed only every 3 days and the increasing concentration of hormone during this period may have inhibited further release. This "mass action" effect has been demonstrated by Kastin et al. (1971) for MSH in pituitary incubations and Schofield (1967) has reported an increased hGH release when the culture medium is changed more frequently (i.e. every hours).

b) Whenever cell structure and hormone production were studied simultaneously in the same culture a good agreement was observed between the morphological and biological findings: a poor secretion is seen when morphological aspects are impaired and necrosis is extensive (Table 1, D), while on the contrary the highest values are observed in experiments in which the tissue remains healthy in appearance (Table 1, A1-C3). Another correlation can be pointed out: hormonal production remains very high over the 2 first weeks in most experiments and then decreases. At the same time explants appear progressively chromophobic and at the 28th day of culture, granules are scanty whereas lysosomes are abundant.

c) The reasons for the disparity of the results in the same experiments are not clear. Indeed on the 14th day, in some cases, the cytological aspect is well maintained and hormonal secretion is still very high (Table 1, B2-B3); in others derived from the same adenoma, a large necrotic area appears and hGH secretion decreases sharply (Table 1, B1). In these cases although the
source of tissue is identical, the surgical damage is not really the same for all fragments: explants derived from the centre of the tumour are undoubtedly less damaged than those from the edge of the tumour.

The size of fragments might also be taken into account, for explants are not strictly identical and it would appear that survival conditions are better in the smaller fragments.

d) Another question concerns the production of proteins other than hGH. When \(^3\)H-leucine is added to the medium, 40% of \(^3\)H-proteins consist of \(^3\)H-hGH but the remaining \(^3\)H-proteins have not been identified. Other pituitary hormones have been assayed occasionally: very low or undetectable MSH, ACTH, FSH and LH levels have been observed which might represent only a small fraction of the \(^3\)H-proteins. Prolactin has not been directly measured in culture medium. Though no prolactin cells have been observed in the numerous series studied, this question remains to be elucidated. Fasteels (1963) has indeed shown how quickly prolactin cells become prominent in anterior pituitary organ culture and a high \(^3\)H-prolactin secretion has been reported to occur in incubation medium from monkey pituitaries after addition of \(^3\)H-leucine (Friesen & Guyda 1971). These experiments have been performed on normal pituitaries. In the incubation medium removed from a somatotrophic adenoma Hwang et al. (1971) observed that after \(^3\)H-leucine incorporation anti-hGH serum precipitated 30 times more radioactive proteins than anti-ovine prolactin serum. From a similar experiment Guyda et al. (1973) obtained as much labelled hGH as labelled human prolactin by immuno-precipitation. These last studies have been performed in short time incubation media. No identical findings have been reported at present in long term organ culture.

ACKNOWLEDGMENTS

Ovine prolactin, NIH-PS 10, used in this experiment, was kindly supplied by the National Institute of Arthritis and Metabolic Diseases (NIAMD).

REFERENCES

Lasnitzki I.: J. Endocr. 30 (1964) 225.

Received on July 3rd, 1974.